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Biosynthesis of Riboflavine in *Corynebacterium* Species: the Purine Precursor

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Corynebacterium species lacks the ability to convert either xanthine or guanine to adenine. This defect and the use of the purine nucleoside antibiotic decoyinine, which blocks the conversion of xanthosine monophosphate → guanosine monophosphate, permit an experimental design in which the interconversion of purines is largely prevented. Cultures of this organism were grown in the presence of decoyinine and various purine supplements. Data obtained by comparing the radioactivity incorporated from guanine-2-^{14}C or xanthine-2-^{14}C into bacterial guanine, xanthine, and riboflavine indicate that guanine or a close derivative of guanine is the purine precursor of riboflavine.

In recent years, a great deal of information has accumulated to support a precursor role for a purine in the biosynthetic sequence leading to riboflavine. The early work of MacLaren gave the first indication of purine involvement. He showed that a number of purine bases stimulated flavinogenesis in cultures of *Eremothecium ashbyii* without affecting growth (16). Similarly, Giri and Krishnaswamy demonstrated that, in a mutant strain of *Saccharomyces cerevisiae*, adenine dramatically increased riboflavine production with no increase in growth (5). Plaut reported studies on the distribution of label from glycine, formate, and carbon dioxide in riboflavine produced by *Ashbya gossypii* (21). He concluded that the pattern of labeling in rings B and C of riboflavine was the same as that previously reported for uric acid from the same precursors (3, 24).

More direct information on purine involvement was obtained by McNutt, who studied the incorporation of variously labeled adenines into the isoalloxazine ring of riboflavine. He concluded that the purine underwent ring opening in the imidazole portion of the molecule, and carbon eight was lost. The remaining diaminopyrimidine was then incorporated into riboflavine (17, 18). The possibility of the purine being degraded to small molecules and their subsequent incorporation into riboflavine via a pathway similar to, but separate from, the de novo purine biosynthetic route could not be excluded. With xanthine totally labeled with ^{14}C and ^{15}N, McNutt unequivocally demonstrated the direct incorporation of the intact diaminopyrimidine moiety remaining after loss of the purine imidazole carbon. The ratio of ^{14}C to ^{15}N was the same in the isolated riboflavine as in the precursor xanthine, providing that a correction was applied for the loss of the imidazole carbon (19). Howells and Plaut recently presented evidence, obtained with a purine-requiring mutant of *Escherichia coli*, that a purine derivative is an obligatory intermediate in riboflavine biosynthesis (9). The precursor role of a purine in this biosynthetic pathway is therefore clearly established, although details of this transformation remain obscure.

A number of investigators have attempted to determine the nature of the purine precursor of riboflavine. On the basis of structural similarities, xanthine or a derivative of xanthine would merit consideration. In growing cultures of *E. ashbyii*, xanthine was shown to be the most efficient purine precursor of riboflavine, with adenine and guanine being less efficient (16). In a mutant strain of *S. cerevisiae*, adenine was found to be by far the most efficient riboflavine precursor (5). Bacher and Lingens (1) recently reported the accumulation of 2,5-diamino-6-hydroxy-4-ribitylamino-pyrimidine in cultures of a riboflavine-deficient mutant of *S. cerevisiae*, and on this basis concluded that a derivative of guanine is the more immediate riboflavine precursor. Facile purine-purine interconversions and possibly different rates of penetration of the various purines tested are among the factors which might explain the obvious ambiguities cited above.

In this paper, we report the results of experiments, carried out in growing cultures of *Corynebacterium* species, designed to circumvent these

difficulties. Decoyinine (9-β-D(5,6-psicofuranoseenyl)-6-amino-purine) is a structural analogue of adenosine, modified in the ribofuranosyl moiety, produced by *Streptomyces hygroscopicus* (4, 8, 27–29). A number of workers have presented evidence that decoyinine inhibits the conversion of xanthosine monophosphate (XMP) → guanosine monophosphate (GMP; 2, 25). Block and Nicol reported that the inhibition of growth of *S. faecalis* by decoyinine could be reversed only by guanine, guanosine, or deoxyguanosine; all three compounds did so competitively (2). Nakayama and Hagino found that xanthine, xanthosine, or XMP was accumulated in large amounts in 23 strains of microorganisms, representing a variety of bacterial species, when cultured in the presence of decoyinine (20).

The organism used in the experiments reported here (*Corynebacterium* species) lacks the capacity to convert guanine or xanthine to adenine. Therefore, the ability to prevent the conversion of xanthine to guanine would provide an experimental design which would permit the study of the incorporation of the individual purines into riboflavine. The use of decoyinine appeared to permit such an experimental design. In *Corynebacterium* species cultured in the absence of guanine derivatives, concentrations of decoyinine above 200 μg/ml completely inhibit growth. This inhibition can be relieved by the addition of guanine or guanosine. Xanthine accumulates and can be isolated from cultures grown in as little as 150 μg of decoyinine per ml. In the experiments reported here, the XMP → GMP pathway was blocked throughout the period of growth. Bacterial growth was therefore dependent upon the addition of a guanine derivative. By taking advantage of the absence of a pathway converting guanine or xanthine to adenine and by using an inhibitor (decoyinine) of the reaction XMP → GMP, experiments have been carried out in growing cultures of *Corynebacterium* species, and these experiments provide evidence that guanine is the purine precursor of riboflavine.

MATERIALS AND METHODS

Materials. Xanthine-*2*-^{14}C was generously provided by Elliott Shaw and was purified by column chromatography before use. Guanine-*2*-^{14}C and Chelex-100 were purchased from Calbiochem, Los Angeles, Calif.

Decoyinine was generously supplied by George B. Whitfield of The Upjohn Co., Kalamazoo, Mich.

Culture medium. The organism was cultured aerobically on a rotary shaker in a stock medium of the following composition, per liter: glucose, 20 g; glycine, 4 g; dipotassium hydrogen phosphate, 0.5 g; potassium dihydrogen phosphate, 0.5 g; ferrous sulfate heptahydrate, 0.04 g; magnesium sulfate heptahydrate, 0.02 g; thiamine, 0.4 mg; and tap water to volume. Stock cultures were carried on slants made by the addition of 2% agar to the above medium (10).

Isolation and purification of riboflavine, xanthine, and nucleic acid purine nucleotides. The above compounds were obtained in impure states by the fractionations schematically outlined in Fig. 1.

Purification of riboflavine. The aqueous acetone eluate from Florisil was concentrated to about 10.0 ml in the dark in vacuo at 35 to 40 C. The concentrate was made 1 N with respect to HCl and was applied to a column (2 by 15 cm) of Dowex 50 H^+. The column was eluted with 0.2 N HCl, and 12-ml fractions were taken. Riboflavine was eluted in fractions 16 to 33. These fractions were pooled, extracted twice with 35 ml of $CHCl_3$ to remove lumichrome if present, and passed over a carefully prewashed column (2 by 2 cm) of Florisil. The Florisil was prewashed with 100 ml of 50% aqueous acetone followed by water. The riboflavine from the pooled fractions became concentrated in a narrow yellow band and was thoroughly washed with water. Riboflavine was eluted in about 20 ml of 50% aqueous acetone. The aqueous acetone was rapidly removed in vacuo, all of the above manipulations being carried out in a darkened room. At this point, spectrally pure riboflavine was obtained (26).

Purification of xanthine. The 3 N NH_4OH eluate was concentrated to dryness in vacuo. The solids were extracted with 30 ml of 1 N HCl and filtered. The filtrate was applied to a column (2 by 15 cm) of Dowex 50 H^+. The column was developed with a linear gradient consisting of 800 ml of water in the mixing chamber and 800 ml of 2 N HCl in the second chamber. Fractions (10 ml) were taken. Spectrally impure xanthine was obtained in fractions 36 to 44. These fractions were pooled, concentrated to dryness in vacuo, taken up in 5 ml of water, and applied to a column (1 by 20 cm) of Chelex-100 in the cuprous form. The column was washed with water (100 ml) and then eluted with 2 N NH_4OH (6); 3-ml fractions were taken. Spectrally pure xanthine was eluted in fractions 10 to 15.

Purification of nucleotides. The bacterial residue was hydrolyzed overnight in 1 N NaOH, and the barium salts of the free nucleotides were obtained by the methods of Rose and Schweigert (22). Purification of the nucleotides was carried out by the procedures of Katz and Comb (12). Samples of the spectrally pure GMP and adenosine monophosphate (AMP) were hydrolyzed to the free bases by refluxing for 30 min in 1 N HCl. Guanine and adenine were purified from the hydrolysates by chromatography on columns (2 by 10 cm) of Dowex 50 H^+. The columns were eluted with 2 N HCl. Guanine was eluted in fractions 23 to 31, and adenine was eluted in fractions 65 to 75; 10-ml fractions were taken.

Radioactive measurements. All radioactive measurements were made with a Nuclear-Chicago Unilux liquid scintillation counter. The counting fluid used throughout had the following composition: naphthalene, 120 g; 2,5-diphenyl-oxazole, 7.0 g; *p*-bis-2′(5-phenyl-oxazolyl)-benzene, 50 mg; *p*-dioxane, to 1 liter. The efficiency of counting was determined for each sample by the channel-ratio method. Specific radioactivities reported were, in all cases, extrapolated

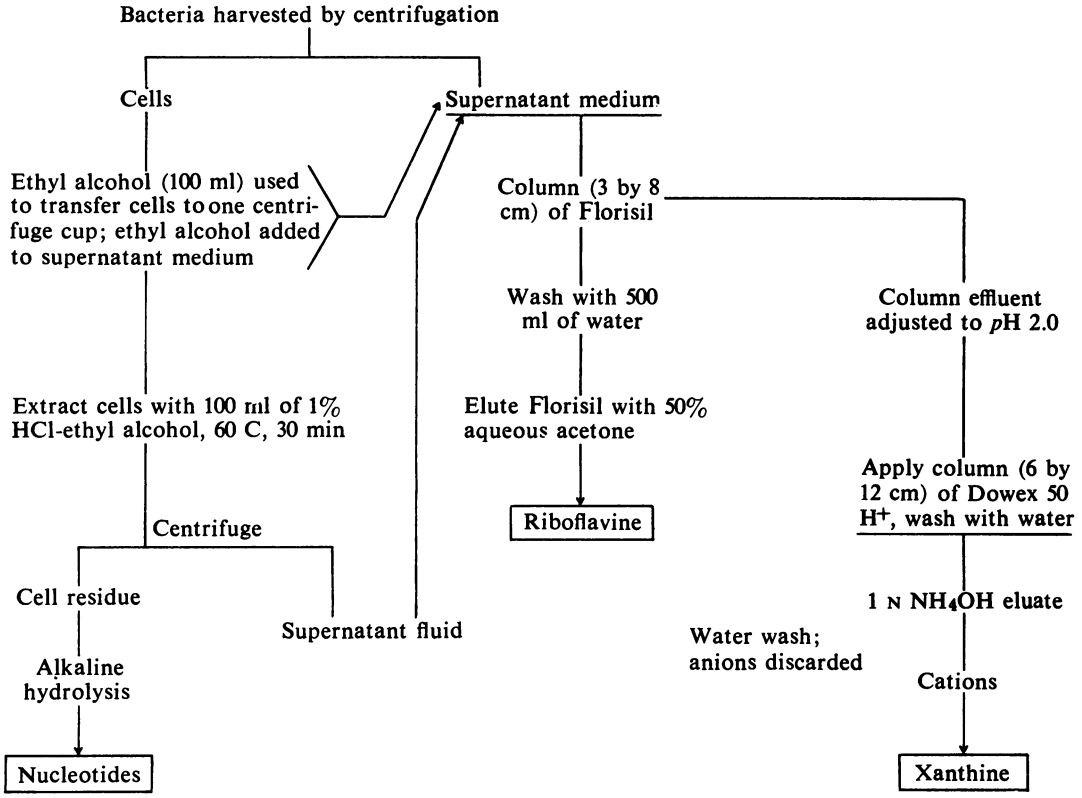

FIG. 1. *Schematic summary of the fractionation of bacterial cells and media to obtain the crude products (boxed).*

to 100% efficiency and are reported as disintegrations per minute (dpm) per micromole.

Bacterial growth was monitored turbidimetrically at 650 nm, with 1-cm square cuvettes and a Beckman DU spectrophotometer. Relative bacterial growth is reported in optical density (OD) units at 650 nm and milligrams (dry weight) per milliliter. One OD unit is equal to 0.3 mg of bacterial dry weight.

RESULTS

Isotope competition experiment. Cultures of *Corynebacterium* species were grown on standard media containing, per liter, 200 μc of uniformly labeled ^{14}C-glucose and 100 μc of uniformly labeled ^{14}C-glycine. Three sets of duplicate flasks were then set up as follows: (i) no competitor added (control); (ii) 200 μmoles of guanine added, and (iii) 200 μmoles of xanthine added. The final volume per flask was 200 ml. The flasks were inoculated and placed on the rotary shaker for 48 hr. Nucleic acid guanine and adenine were isolated from all flasks. The nucleic acid adenine radioactivity was the same in all flasks, 12,000 dpm/μmole (Table 1). The guanine specific radioactivity fell from 12,000 dpm/μmole in the control flasks to 700 dpm/μmole (5.8% of the control)

TABLE 1. *Isotope competition experiment*

Nonradioactive competitor[a]	Adenine (dpm/μmole)	Guanine (dpm/μmole)	Per cent of control	
			Adenine	Guanine
None (controls)	12,000	12,000	100	100
Guanine	12,000	700	100	5.8
Xanthine	12,000	5,000	100	41.8

[a] Concentration of competitor, 1 μmole/ml.

in the case of the guanine-supplemented flasks and 5,000 dpm/μmole (41.8% of the control) for the xanthine-supplemented flasks. The inability of *Corynebacterium* species to convert either xanthine or guanine to adenine was therefore established, since in all cases the incorporation of the label from ^{14}C-glucose and ^{14}C-glycine into nucleic acid adenine was not affected by supplementation with nonradioactive guanine or xanthine. Additional support for this defect may be seen in Tables 2 and 3.

Decoyinine-xanthine-2-^{14}C experiment. Fourteen 500-ml Erlenmeyer flasks were charged with

regular *Corynebacterium* species medium. The following supplements were added, per milliliter: 1.0 μmole of guanosine, 0.05 μmole of xanthine-2-^{14}C (specific activity, 231,450 dpm/μmole), and 600 μg of decoyinine. The guanosine and decoyinine were added aseptically after sterilization by filtration. Xanthine was sterilized by autoclaving in the media for 10 min at 120 C. In all other respects, the media and growth conditions were essentially as previously described. The final volume per flask was 100 ml. The concentration of decoyinine used was twice the amount required to inhibit growth of cultures of *Corynebacterium* species having an OD of 6.0 units/ml at 650 nm (1.8 mg, dry weight/ml). The flasks were inoculated by the addition of 1.0 ml of inoculum from a culture growing in the logarithmic phase and were placed on a rotary shaker at room temperature. After 96 hr on the shaker, the cultures reached an OD of 3.6 units/ml at 650 nm (1.08 mg, dry weight/ml) and were harvested. Riboflavine, xanthine, and GMP were isolated and purified by the procedures described above. Guanine and adenine were obtained from bacterial nucleotides as described. The relative specific radioactivities of these compounds are tabulated in Table 2.

Decoyinine-guanine-2-^{14}C experiment. Two modifications in the experimental design were made. First, guanine (1 μmole/ml) was used in place of the guanosine employed in the previous experiment. This change was necessary because of difficulties in obtaining guanosine-2-^{14}C; it was not considered crucial to the experimental design, since it had been reported (2) and confirmed in this laboratory that either guanine or guanosine will bypass the decoyinine inhibition and permit bacterial growth. Second, the concentration of decoyinine was reduced from 600 to 400 μg/ml. This change in decoyinine levels was made because the inhibition of bacterial growth in the decoyinine-^{14}C-xanthine experiment was greater than expected, and thus an inordinate amount of incubation time was required in order to obtain adequate bacterial growth and an amount of riboflavine which could be isolated.

Fourteen 500-ml Erlenmeyer flasks were set up as described in the decoyinine-^{14}C-xanthine experiment. The supplemental additions were 1.0 μmole of guanine-2-^{14}C per ml and 400 μg of decoyinine per ml. The guanine was autoclaved with the media, and the decoyinine was added aseptically after sterilization by filtration. The final volume per flask was 100 ml. Inoculation was as described above in the decoyinine-xanthine experiment. After 21 hr of incubation, the cultures reached an OD of 5.8 units/ml at 650 nm (1.74 mg, dry weight/ml). The cells were harvested and fractionations were carried out as described above. The relative specific radioactivities are shown in Table 3.

DISCUSSION

The inability of *Corynebacterium* species to convert xanthine or guanine to adenine and the use of an inhibitor of XMP → GMP conversion (decoyinine) have permitted in vivo experiments designed to ascertain the nature of the purine precursor of riboflavine. The data obtained from such experiments and presented here are interpreted as a clear indication that guanine or a close derivative of guanine is the purine precursor of this vitamin.

A very active guanine aminohydrolase present in *Corynebacterium* species could explain the radioactivity in the isolated xanthine in the decoyinine-guanine-2-^{14}C experiment (Table 3). The fact that xanthine in this experiment is not closer to the added guanine in its specific activity and the fact that the specific activity of isolated xanthine was greatly reduced with respect to the starting material, in the decoyinine-xanthine-2-^{14}C experiment (Table 2), reflect the considerable accumulation of nonradioactive, de novo-synthesized xanthine which resulted from the decoyinine block. A further indication of the large xanthine accumulation in decoyinine-inhibited cultures is apparent when the inability to isolate

TABLE 2. *Summary of data from the decoyinine-xanthine-2-^{14}C experiment*

Compound	Specific radioactivity (dpm/μmole)
Riboflavine	2,758
Nucleic acid GMP	2,565
Nucleic acid guanine	2,159
Final xanthine	12,225
Nucleic acid adenine	<10
Initial xanthine-2-^{14}C	231,450

TABLE 3. *Summary of data from the decoyinine-guanine-2-^{14}C experiment*

Compound	Specific radioactivity (dpm/μmole)
Riboflavine	100,690
Nucleic acid GMP	86,865
Nucleic acid guanine	76,276
Final xanthine	23,248
Nucleic acid adenine	<10
Initial guanine-2-^{14}C	114,500

xanthine from *Corynebacterium* species grown on standard media is considered. Repeated attempts to isolate xanthine from cultures of this organism met with failure. In contrast, large amounts of xanthine accumulate and can be readily isolated from decoyinine-inhibited cultures.

Although alternative explanations are possible, the close agreement between the specific radioactivities of the exogenous guanine-2-^{14}C and the isolated riboflavine (Table 3) suggests that the free-base guanine is a more direct purine precursor of riboflavine than either GMP or guanosine triphosphate (GTP). The data presented do not exclude the possibility that the guanine precursor of riboflavine is not in equilibrium with the GTP pool that ultimately gives rise to the ribonucleic acid GMP.

It should be pointed out that riboflavine was isolated from both the bacteria and the culture medium, whereas GMP and guanine were isolated from the cellular nucleic acids. The higher riboflavine specific activity (Table 3) relative to nucleic acid GMP could conceivably be the consequence of excretion of riboflavine while nucleic acid GMP is diluted by de novo-synthesized GMP.

The involvement of guanine or a derivative of guanine in this biosynthetic pathway raises an interesting point relative to the biosynthesis of folic acid and the azapteridine antibiotic toxoflavin. It is now well established that GTP is a direct precursor of the pteridine moiety of folic acid (11, 13, 17, 23). Similarly, GTP has been demonstrated to be the purine precursor of the azapteridine, toxoflavin (14, 15). The possibility of one or more common intermediates in the biosynthesis of these two vitamins and the antibiotic toxoflavin is apparent.

In summary, it is clearly established that a purine, after loss of its imidazole carbon, is incorporated intact into the isoalloxazine ring of riboflavine (19). It has also been demonstrated that a purine is an obligatory intermediate in this pathway (9). We here extend these findings to exclude adenine and xanthine. Furthermore, we present data to indicate that guanine or a close derivative of guanine is the purine precursor of riboflavine. These results are in agreement with those of Bacher and Lingens (1).

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